Breaking the chains: deubiquitylase specificity meets function.

Michael J. Clague[1], Sylvie Urbé[1], David Komander[2]

[1]Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, UK; [2]Ubiquitin Signalling Division, The Walter and Eliza Hall Institute of Medical Research, Melbourne , Australia.

email: clague@liv.ac.uk, urbe@liv.ac.uk, dk@wehi.edu.au

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Abstract

The deubiquitylase (DUB) family of enzymes maintains the dynamic state of the cellular ubiquitome by releasing ubiquitin from proteins. Accordingly, DUBs occupy key roles in almost all aspects of cell behaviour. Many DUBs show selectivity for particular linkage-types or positions within ubiquitin chains. Others show chain-type promiscuity, but select a distinct palette of protein substrates via interactions, established through binding modules outside the catalytic domain. The ubiquitin chain cleavage mode or chain linkage specificity has been related directly to biological functions. Examples include proteasomal ubiquitin recycling, DNA repair pathways and innate immune signaling. DUB cleavage specificity is also being harnessed for analysis of ubiquitin chain architecture. The recent development of highly specific DUB inhibitors heralds their emergence as a new class of therapeutic targets, linked to numerous disease states.

Introduction

Conjugation of the 76 amino acid polypeptide ubiquitin to substrate proteins is a reversible post-translational modification, involved in the regulation of most cellular processes. The ubiquitin system may be considered as the complement of proteins that convert free ubiquitin molecules to a complex code written upon 1000s of different substrate proteins [1-4]. The net ubiquitylation status of the cell reflects the combined activities of several hundred ubiquitin conjugating enzymes (E1, E2 and E3s), counterbalanced by 99 currently identified deubiquitylases (or deubiquitinases, hereafter DUBs). The ubiquitin system has two main outputs: control of protein turnover by providing proteasomal and lysosomal targeting signals and governance of cell signaling networks by regulation of protein interactions and activities, akin to phosphorylation. Thus, the balance between ubiquitylation and deubiquitylation is tightly coupled to the regulation of protein levels and activity. DUBs also maintain cellular ubiquitin levels by processing newly synthesised ubiquitin molecules and reclaiming ubiquitin from proteins destined for degradation (Figure 1). The DUBs are currently drawn from 7 evolutionarily conserved families, two of which (MINDY and ZUP1) have been discovered only recently (Figure 2).

Ubiquitylation most commonly occurs at lysine residues of substrate proteins. Importantly ubiquitin's seven internal lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) allow for the generation of isopeptide linked ubiquitin chains of diverse architecture and length. Ubiquitin derives from 4 genes that code for linear fusion proteins incorporating one or more ubiquitin molecules, from which free ubiquitin is generated by DUB cleavage of the peptide bond [5]. Linear or Met1-linked ubiquitin chains can also be assembled enzymatically from single ubiquitin moieties, through a unique E3 ligase complex known as the linear Ub chain assembly complex (LUBAC) [6]. Further complexity is provided by post-translational modification of ubiquitin (e.g. phosphorylation, acetylation) and by linking to other ubiquitin-like molecules (e.g. SUMO, NEDD8, ISG15). These complex patterns constitute a "ubiquitin code", which is read by hundreds of proteins that incorporate ubiquitin binding domains [1,7].

In a typical mammalian cell, more than half of total ubiquitin is represented by single ubiquitin molecules conjugated to lysine residues in the substrate (mono-ubiquitylation) [2,8]. A further 10-20% of ubiquitin is incorporated into chains, for which the representation of each linkage type varies between cell types and cell states [2,8]. Accordingly, DUBs handle ubiquitin modifications in two fundamentally distinct manners. Many are directed towards specific protein substrates via protein interaction domains distinct from the catalytic domain (catalogued in previous reviews [9,10]). Other DUBs recognise and show selectivity for particular ubiquitin chain architectures and may not be able to remove the proximal ubiquitin molecule that is directly attached to the protein (recently reviewed in [11]). Linkage selectivity can either be encoded within the catalytic domain, or conferred through co-operation with ubiquitin binding domains within DUBs or their interaction partners.

Here, we will focus on recent advances in understanding the physiological functions of DUBs, emphasising examples where selectivity towards particular ubiquitin chain architectures connects with defined cellular roles, e.g. in DNA repair, cell cycle and innate immune signaling pathways. We also discuss how selective DUBs can provide analytical tools for investigation of ubiquitin chain architecture and conclude by highlighting recent advances spurring their development as therapeutic targets.

DUB families

Six of the seven families of DUBs (USPs, UCHs, OTUs, MJDs, MINDYs, ZUP1) are classified as cysteine proteases, whilst the JAMM/MPN family are zinc-dependent metalloproteases. With the exception of the MJDs each family is conserved from yeast to humans (Figure 1). Eleven of ninety-nine family members are considered to be pseudoenzymes, in that they have lost residues critical for DUB activity, but can nevertheless perform vital functions [12]. This is particularly common in the twelve member JAMM family, which contains five pseudoDUBs. The phylogenetic relationships and domain structures of the five longest established families (USP, OTU, Josephin (MJD) , UCH and JAMM) have been covered extensively elsewhere [9,10]. Two new families of DUBs have recently been discovered. The MINDY family has two members in *S. cerevisiae*, and is expanded to five in humans, including one pseudoDUB [13]. Little is known about cellular function of this family, but each member tested to date shows specificity for Lys48 linked ubiquitin chains, strongly indicating roles in protein homeostasis [14]. The human genome contains one representative of the ZUP1 family, whose specificity for Lys63-linked chains is conferred by multiple ubiquitin binding domains and which has been linked to genome maintenance pathways [15-18]. Figure 1 provides an updated overview of DUB conservation across species from yeast to man.

DUB specificity

DUBs are proteases which cleave peptide or isopeptide bonds between conjoined ubiquitin molecules or between ubiquitin and a modified protein. The complexity of ubiquitin chain architectures dictates a wide variety of distinct DUB activities and preferences (Table 1)[11]. Adjacent ubiquitin molecules, within a chain, are not equivalent: throughout, we will refer to the "distal" ubiquitin, as that which presents its C-terminal glycine to the DUB active site and which links to a "proximal" moiety via the scissile bond. Aside from discriminating chain linkage type, DUBs may choose between processing from the distal end, gradually chewing down the chain (exo-DUB activity), or cleaving within chains (endo-DUB activity). Chain length provides another variable, with some DUBs preferring longer chain types (e.g. MINDY, OTUD2 and ATXN3) [13,19,20]. Others will specialise in the cleavage of monoubiquitin from specific protein substrates (e.g.

histone directed DUBs, see below) or clipping off an intact ubiquitin chain (*en-bloc* cleavage, e.g. proteasomal DUBs, see below). Three enzymes containing DUB catalytic domains were later shown to specifically target ubiquitin like molecules; USPL1 is a SUMO protease [21], USP18 is an ISG15 specific protease [22] and the COP9 signalosome component CSN5 targets NEDD8 [23]. The members of the OTU family display diverse chain preferences and their study unveiled many principles of DUB chain linkage specificity [11,20,24]. In contrast, systematic studies of USP family members showed orders of magnitude differences in catalytic turnover but only modest ubiquitin chain preferences [25,26]. However, a sub-set of USP enzymes, including USP30 and CYLD show marked chain preferences that are encoded in their catalytic domains (Table 1) [27-29]. Despite a wealth of structural information (reviewed in [11]), prediction of linkage or substrate specificity remains challenging and needs to be determined biochemically.

Counting and mapping DUBs

To understand the impact of individual DUBs on cellular processes, both individual protein copy numbers and location are important considerations. Mass spectrometry derived data sets can provide global protein copy number estimates. For DUBs, the estimated range covers several orders of magnitude from low hundreds (limit of detection) to hundreds of thousands per cell for the most abundant enzymes [2]. Available data suggest that high copy number DUBs perform broad "housekeeping" functions (e.g. proteasomal DUBs) whilst the rarer forms have more specialist roles. Several linkage specific DUBs are highly represented including OTUB1 (Lys48), OTUD7B (Cezanne; Lys11) and OTULIN (Met1). Some of these (e.g. OTULIN) may globally suppress the accumulation of ubiquitin chains bearing these linkages [30]. In practical terms, this would effectively suppress the background noise, against which a specific or localised signal can emerge.

Multiple approaches have been used to determine the sub-cellular distribution of DUBs (Figure 2). Systematic mapping of GFP-tagged DUBs, using fluorescence microscopy in mammalian cells, has allowed the broad classification of DUBs with predominantly cytosolic or nuclear localisation [31]. A sub-set of enzymes show specific association with a variety of defined structures including nucleoli (USP39), microtubules (USP21) and the plasma membrane (USP6). Two DUBs, USP19 and USP30 possess trans-membrane domains and show distinct localisations to the endoplasmic reticulum (ER) or mitochondria and peroxisomes, respectively [32,33]. This system-wide approach has been extended to screen for DUBs which translocate following a specific cellular perturbation (e.g. DNA damage [34]). An orthologous approach is to combine sub-cellular organelle fractionation with quantitative mass spectrometry, which has the further advantage of providing an estimate of the protein copy number associated with each organelle [35]. Detailed studies of individual DUBs have also revealed locations which were not captured in global screens. For example several additional DUBs have recently been added to the

complement of centrosomal DUBs (USP21, USP33, USP9X) [36-39]. Figure 3 provides a synthesis of currently available data. Numerous DUBs are produced as multiple splice variants, which localise to different compartments and may turnover at different rates. Interesting examples include, USP19 which localises to the Endoplasmic Reticulum (ER) or cytosol depending on the presence of a trans-membrane domain [32,40], USP33 which localises to the ER and Golgi [41] and USP35 for which one form localises to the ER and to lipid droplets and others to the cytosol [42]. A short form of USP35 has also been linked to mitochondria, but this variant lacks an intact catalytic domain [43].

Cellular functions of DUBs

The essential DUBs.

The introduction of whole genome based CRISPR/Cas9 screens for viability across large numbers of cell lines has generated an overview of those DUBs that are required across multiple cell types i.e. represent core fitness genes [44,45]. The collated results of major studies are presented in Table S1. The essential DUBs are widely expressed in high copy numbers [2]. Three pairs of proteins stand out, each of which are embedded within ancient multi-molecular complexes (Figure 4). Two pseudo-DUBs from different families, USP39 and PRPF8 are components of the spliceosome complex involved in pre-mRNA splicing at the nucleolus. PRPF8, a large protein, is remarkable in containing no less than 4 pseudoenzyme domains showing homology to restriction endonuclease, reverse transcriptase and RNAaseH in addition to an inactive JAMM domain. The JAMM family members COPS5 and COPS6, active and inactive respectively (also called an MPN^+:MPN unit), cooperate within the core of the eight sub-unit COP9 signalosome, to remove the ubiquitin like molecule NEDD8 from Cullins and thereby inactivate Cullin-RING E3 ligases (CRLs) [23]. An essential DUB module comprising a further MPN^+:MPN combination, PSMD14 and PSMD7 (Rpn11 and Rpn8 in yeast), is involved in substrate processing by the proteasome (see next section and Figure 4) [46].

USP5 is the most abundant of a set of DUBs (including USP3, USP13, USP16, USP22, USP33, USP44, USP45, USP49) that bear zinc finger ubiquitin binding domains (ZnF-UBP), which in some (e.g. USP3, USP5, USP16) but not all cases (e.g. USP13, USP22, USP33) has been shown to recognise the carboxyl-terminal Gly-Gly motif of unattached ubiquitin [47,48]. This confers the capacity to specifically recognise free ubiquitin chains, which may be derived from newly synthesised linear ubiquitin or from chains that have been removed from substrates *en bloc.* Thus, USP5 is a core fitness protein by virtue of suppressing the accumulation of unattached ubiquitin chains and maintaining levels of monoubiquitin, the essential currency of the ubiquitin economy. Its activity against free chains has also recently been proposed to promote the disassembly of heat induced stress granules [49]. USP36 is a prominent nucleolar DUB and most likely contributes to cell viability by governing the stability of RNA polymerase 1 and consequent

ribosome biogenesis [50]. Some of the other DUBs that are also widely required for cell viability (USP7, USP8 collated in Table S1) are mentioned elsewhere in this article, others are less well studied, such as the SUMO specific USPL1 [21,51].

Proteasomal DUBs and *en bloc* ubiquitin chain cleavage

Ubiquitin was first linked to protein degradation through elucidation of its role as a proteasome targeting signal [52]. The 26S proteasome consists of a barrel shaped core particle (20S) capped at one or both ends by a 19S regulatory particle. The 19S regulatory particle provides a binding platform for ubiquitin and co-ordinates entry into the 20S core particle where proteins are degraded. It is now clear that multiple types of ubiquitin chains, including branched architectures, provide efficient proteasomal targeting signals [53-56]. Three catalytically active DUBs from distinct families, USP14, UCHL5 and PSMD14, are associated with the lid of the 19S regulatory particle and co-ordinate essential proteasomal substrate pre-processing [57].

For protein degradation to occur, a substrate must be unfolded to thread into the catalytic chamber of the 20S particle. Attached ubiquitin provides a barrier to translocation and must be removed. The JAMM family member, PSMD14, sits directly on top of this entry portal that is comprised of a hexameric ring of AAA-ATPases [58,59]. Purified proteasomes lacking this DUB activity are deficient in protein degradation. A current model maintains that for substrates committed to entering the catalytic chamber, attached ubiquitin chains are mechanically drawn to the entry port by concerted ATPase activity of 19S associated AAA-ATPase proteins and thereby encounter the catalytic site of PSMD14, followed by hydrolysis of the isopeptide bond at the substrate lysine [46,60-62]. Although PSMD14 itself neither binds nor hydrolyses ATP, its DUB activity is indirectly ATP-dependent by virtue of this coupling [63,64].

PSMD14 forms a dimer with the MPN family member pseudo-DUB, PSMD7. Isolated PSMD14/PSMD7 heterodimers show little ubiquitin linkage specificity *in vitro* [60]. However once incorporated into the regulatory particle, steric inhibition by components of the entry portal precludes di-ubiquitin spanning the catalytic centre. This ensures that ubiquitin chains are removed *en bloc* as only the isopeptide bond between the substrate lysine and the C-terminus of the first ubiquitin can be hydrolysed [64]. The active site organisation of PSMD14 is similar to the endosomal DUBs AMSH and AMSH-LP (see also below). However, these proteins have stringent specificity for Lys63 ubiquitin chains, conferred by an insertion loop in the catalytic domain (Ins-2 loop) that enables binding to the proximal ubiquitin [65]. The equivalent loop in PSMD14/Rpn11 serves to anchor the protein within the proteasome [46,60].

When ubiquitylated proteins first bind to the proteasome they are not yet committed to degradation. That step is believed to require presentation of a constitutively or transiently unfolded region to the ATPase machinery [57]. In distinction to PSMD14, USP14 and UCHL5 (also known as UCH37) are not integral components of the proteasome. They bind to lid components

PSMD11 (Rpn6 in yeast) and RPN13 respectively, which leads to their activation [66-69]. Neither DUB represents an essential gene, with UCHL5 completely lacking in *S. cerevisiae*. Rather than coupling to degradation, the combined activities of USP14 and UCHL5 may offer a reprieve from degradation by releasing proteins from the proteasome before the AAA-ATPase motor has engaged.

USP14 may also play a positive role in protein degradation by pre-processesing certain proteasome substrates in an interesting way, which has been elucidated using Cyclin B as a model. The APC/Cyclosome (APC/C) ubiquitylates Cyclin B with multiple chain types spread across the disordered N-terminus of the protein, to provide an efficient proteasomal degradation signal [68,70]. Deubiquitylation of Cyclin B by proteasome associated USP14 is rapid and ATP-independent. Reducing the number of ubiquitylation sites on Cyclin B revealed that USP14 shows a marked specificity for a substrate with multiple chains attached, irrespective of tested chain linkage types. When faced with Cyclin B bearing multiple tetraubiquitin chains, two surprising results were found. The cleavage reaction yields intact tetraubiquitin chains i.e. cleavage occurs *en bloc,* as discussed above for PSMD14. Secondly, the reaction yields a substrate with a single residual tetraubiquitin chain attached [68]. Therefore, in the case of a multi-ubiquitylated substrate USP14 and PSMD14 appear to function in series. USP14 strips off supernumerary ubiquitin chains in order to relieve the burden on PSMD14, which must compete effectively with protein unfolding activities.

Substrates of UCH family proteins are restricted according to leaving group size by a flexible active site cross-over loop (ACL), characteristic of this family [71]. When Ub-AMC, a fluorescent substrate presenting a small leaving group, is provided, UCHL5 is the most active proteasomal DUB [72,73]. However, it shows poor activity towards ubiquitin-protein conjugates and homotypic ubiquitin chains of any linkage type [54,68]. 19S Regulatory particle associated UCHL5 can trim chains from the distal end irrespective of linkage type, but the slow time scale brings into question the physiological relevance of these findings [74]. It has been proposed that specific substrates may be sufficiently flexible to loop through the ACL [69]. If so, this would again result in *en bloc* ubiquitin chain removal.

The identity of physiologically relevant substrates of UCHL5 and other UCH enzymes remains an open question. Interestingly, UCHL5 moonlights as part of the chromatin remodeling complex INO80 that functions in transcription and DNA repair (see also below for discussion of DUBs in DNA repair) [34,75]. In fact, CRISPR/Cas9 cell viability screens across multiple cell lines reveal that sensitivity of particular cell lines to loss of UCHL5, correlates with the loss of other components of this complex [76]. Structural studies have uncovered a role for UCHL5 interaction with DEUBAD domains in the regulation of its catalytic activity and that of a related family member, BAP1 [67,69,77,78]. In the case of UCHL5, its respective interactions with the DEUBAD domains in RPN13 and the INO80 sub-unit NFRKB, have opposite effects [77]. RPN13 DEUBAD

activates UCHL5 whilst the NFRKB DEUBAD domain blocks ubiquitin binding and thereby acts as a DUB inhibitor [67,69].

Nuclear DUBs act on multiple chain types to regulate chromatin and DNA repair.

In a fluorescence screen of 66 GFP-tagged DUBs, 12 were found to be exclusively nuclear and a further 16 exclusively cytoplasmic in asynchronous HeLa cells [31]. Thus, a large fraction of DUBs experience the nuclear environment, where they can influence genome surveillance and repair pathways, epigenetics/chromatin organisation and transcription.

In HEK293 cells it is estimated that around 60% of conjugated ubiquitin is in the form of monoubiquitin, about half of which is associated with the histone enriched fraction [8]. About 5-15% of histone H2A is mono-ubiquitylated, principally at Lys119, making H2A the most abundant ubiquitylated protein in the cell. Ubiquitin is therefore a major post-translational component of the histone landscape, that influences chromatin structure and function, together with methylation and acetylation. Early pulse-chase studies showed that this modification on H2A has an average half-life of ~90 minutes in HeLa cells, inferring histone directed DUB activity [79]. As described below, at least 6 DUBs have now been linked to histone deubiquitylation (Figure 5).

MYSM1 is the only mammalian DUB that has clear chromatin binding domains, incorporating both SWIRM (Swi3p, Rsc8p and Moira) and SANT (SWI-SNF, ADAN-CoR, TFIIIB)/Myb domains. Accordingly, it is one of several DUBs linked with histone deubiquitylation alongside BAP1, USP3, USP16 and USP22 [80-83]. The three USPs possess a ZnF-UBP domain, N-terminal to their catalytic domain. In USP3 and USP16, this domain recognises the free C-terminus of ubiquitin (see above) and can act as a free ubiquitin sensor in the nucleus, but it may also recognise as yet unidentified chromatin components or associated factors [48]. For example histone H4 has a C-terminal diGly motif in common with ubiquitin.

USP22 is a component of the Spt-Ada-Gcn5-acetyltranserase (SAGA) complex responsible for deubiquitylation of H2B-Ub (see below). In this case its ZnF-UBP domain does not recognise free ubiquitin, but is instead used to make interactions with other SAGA complex components that are required for its activation [48,84]. The UCH family member and tumour suppressor, BAP1, is commonly mutated in certain cancer types [85]. For deubiquitylation of Ub-H2A, it requires activation by interaction with ASXL, recalling the activation of UCHL5 by RPN13 described above [77,78,86]. BAP1 and ASXL proteins together form the Polycomb repressive deubiquitinase (PR-DUB) complex that sits on Polycomb group target genes and maintains silencing of a particular sub-set of genes [83]. The complex is specific for mono-deubiquitylation at Lys119 and cannot remove DNA damage-dependent ubiquitylation of H2A at Lys13 or 15 (see below)[78].

The study of DUBs in DNA damage repair pathways has been particularly intensive 87. The first shRNA screen across the DUB family identified USP1 as the DUB that removes

monoubiquitin from Fanconi anaemia group D2 protein (FANCD2), a key protein involved in the Fanconi Anaemia DNA cross-link repair pathway [88]. USP1 similarly deubiquitylates the DNA processivity factor PCNA, in order to curb the error prone translesion synthesis repair pathway [89]. Global proteomics studies have revealed thousands of ubiquitylation events as part of the DNA damage response to UV and ionising radiation, coupled to an enigmatic bulk increase in Lys6 and Lys33 chains [90]. Accordingly, a multi-parametric screen of DNA damage signatures, alongside numerous other studies has associated many DUBs with this response [34] (Figure 5).

Ionising radiation induced DNA double strand breaks (DSBs) lead to recruitment of an RNF20/RNF40 E3 ligase heterodimer at the site of damage, resulting in monoubiquitylation of H2B at Lys120. This is believed to initiate chromatin opening, which then allows access for repair factors [91-93]. Subsequent deubiquitylation at this site has been attributed to USP22, acting within the SAGA complex [91]. Its activity is required for optimal phosphorylation of Histone H2AX (denoted γ-H2AX) [94]. Lys63-linked chains are both abundant at DNA damage sites and required for double strand break repair [95]. The E3 ligase RNF8 is recruited to phosphorylated γ-H2AX, where it can generate Lys63 chains on linker histone H1 or on the RNF168 interacting protein, L3MBTL2 (lethal(3) malignant brain tumour like protein 2) [96,97]. Lys63 chains at repair sites serve to recruit a second E3, RNF168, which promotes mono-ubiquitylation of H2A at Lys13/15 and further Lys63 polyubiquitylation [98]. Following DNA damage, the normally short-lived RNF168 is itself stabilised through the activity of USP34, which is recruited to damage sites [99]. The Lys13/15 monoubiquitylation signal on H2A partially determines the recruitment of 53BP1, a critical step in initiation of the non-homologous end joining DSB repair (NHEJ) pathway [100]. USP51 has been shown to specifically reverse this signal and thereby regulate DNA damage repair [101]. Lys63-linked chains also mediate recruitment of the BRCA1-A complex (see below). RNF8 further co-operates with UBE2S to generate Lys11 chains on H2A. This promotes transcriptional silencing associated with DNA repair, and is antagonised by the Lys11 specific DUB, Cezanne [102]. Although it is nominally a Lys48 specific DUB, the highly abundant OTUB1 limits Lys63 chains in the DSB repair pathway by binding to and inhibiting transfer from the ubiquitin-charged E2 enzyme Ubc13 [103-105].

The JAMM family member, BRCC36, functions within the BRCA1-A complex consisting of RAP80, BRCC45, MERIT40 and ABRAXAS1 (FAM175A), to which BRCA1 is associated in a phosphorylation-dependent manner. ABRAXAS1 contains a MPN like domain that is not itself catalytically active. The MPN$^+$:MPN related heterodimer of BRCC36 and ABRAXAS1 is likely to constitute the minimal active enzymatic unit, recalling other such couples already described above (see also Figure 4) [12]. BRCA1-A is recruited to sites of DSB sites by RAP80 binding to Lys63-linked chains. Chain selectivity of this complex is stringent towards Lys63 and hence provides exquisite feedback control to limit the RNF8 ubiquitin signal. BRCC36, BRCC45, Merit40 proteins also form a cytosolic complex with an ABRAXAS1 paralogue, ABRAXAS2/KIAA0157 and

adaptor protein SHMT2, collectively known as the BRISC complex (BRCC36 isopeptide complex), which has been linked to stabilisation of type 1 interferon receptor [106].

BRCA1 itself, promotes DNA end resection to produce the ssDNA necessary for homology directed repair (HDR). Its N-terminus associates with BARD1 to generate an active E3 ligase that ubiquitylates H2A at Lys 125/127/129 and promotes resectioning, which can be reversed by USP48 [107-109]. It also interacts with PALB2 to recruit additional repair factors, BRCA2 and RAD51, to DSB sites. Of note, HDR is only active in cells in the S and G2 phases of the cell cycle, due to the requirement for the homologous DNA template. Accordingly, in G1 cells, repair factor recruitment is suppressed by ubiquitylation of PALB2 that can be counteracted by USP11 in a cell-cycle dependent manner [110].

The recently discovered ZUP1 DUB, which exhibits Lys63 specificity, interacts with the replication protein A (RPA) complex, which plays a critical role in the HDR and replication stress pathways by demarcating single strand DNA regions that are generated [15-18]. Available data have not been able to functionally link ZUP1 to DSB repair pathways. However, ZUP1 depletion in cells with elevated ssDNA resulting from replication stress (e.g. hydroxyurea treatment) leads to enhanced micronuclei generation indicative of chromosome instability [15,18].

DUBs and innate immune receptor signalling (linear and Lys63 chains).

Many receptor initiated signaling cascades are now known to utilise the ubiquitin code. This concept was first established from studies of innate immunity and the central NF κ B signaling pathway, which invoked the requirement or Lys63 linked chains [111]. This pathway has continued to provide fresh insight, including clearly defined roles for chain-specific DUB activities. It is now appreciated that innate immune signaling mediated by pattern recognition (e.g.TLR4, NOD2) or cytokine receptors (e.g. TNFR and IL-1R) involves the assembly/disassembly of both Met1, and Lys63 chains on components of the receptor signaling complexes (Figure 6).

The activated pattern recognition receptors and cytokine receptors recruit adaptor proteins including Receptor Interacting Protein Kinases (RIPK1 or RIPK2), Myeloid Differentiation Primary Response 88 (MyD88) or Interleukin 1 Receptor Associated Kinases (IRAKs). Their modification with Lys63 Ub chains serves as a recruitment platform for the TAB1/TAK1 kinase complex, an initiator of multiple kinase cascades [112]. Ubiquitylation of receptor adaptors also promotes recruitment of the Met1 specific E3-ligase LUBAC [113,114]. This results in assembly of Met1 ubiquitin chains on the adaptors directly, or on existing Lys63 chains leading to branched or hybrid chains [113,115-117]. These Met1 chains mediate downstream signaling by interaction with the inhibitor of nuclear factor kappa-B kinase (IKK) sub-unit NEMO [118]. Co-localisation of TAK1 and IKK leads to IKK activation. This then activates a cascade leading to ubiquitylation and degradation of inhibitor (I κ B) proteins, which allows NF - κ B to enter the nucleus and turn on target genes involved in immune and inflammatory responses [112].

OTULIN, a stringent Met1 linkage specific DUB, binds to the PUB domain of the LUBAC component HOIP [117,119-122]. CYLD, belonging to the USP family and specific for Lys63 and Met1 ubiquitin chains [123,124], can also indirectly bind to the same domain on HOIP through an adaptor protein, SPATA2 [125-128]. Both DUBs can restrict NF κ B signaling, but only OTULIN controls the accumulation of linear ubiquitin on LUBAC components and thereby maintains their protein stability [30,117,119,129-135]. In addition, depletion of OTULIN, but not CYLD, leads to a dramatic increase of steady-state Met1 chain levels in cells [30,120,134,136] suggesting that OTULIN is essential for globally restricting Met1 chain accumulation, and implying a more specialised role for CYLD.

The binding of OTULIN and CYLD to LUBAC is mutually exclusive [125,133], suggesting that they regulate distinct aspects of signalling. Indeed, OTULIN is not stably associated with the NOD2 or TNFR1 complexes [133], although its recruitment to TNFR1 has been observed by mass spectrometry [128]. In contrast, SPATA2-CYLD is stably recruited to both NOD2 and TNFR1 via HOIP [133]. There is evidence that OTULIN limits Met1 chain accumulation on receptor-associated adaptors associated with TNFR1 and NOD2 receptors; absence of OTULIN leads to enhanced ubiquitylation of adaptors without changing the overall banding pattern of ubiquitylated forms as judged by Western blotting [117,119,134]. In contrast, depletion of CYLD leads to the accumulation of higher molecular weight forms [134], consistent with CYLD being the major regulator of the length of Met1 and Lys63 chains at these receptor complexes [133].

OTULIN and CYLD are directly linked with human pathologies. CYLD truncations cause cylindromatosis characterised by the formation of benign tumours on the skin of the head and neck [137]. Mutations in OTULIN that ablate or severely reduce activity, cause OTULIN-Related Autoinflammatory Syndrome (ORAS), also known as Otulipenia, which is characterised by neonatal onset fevers, skin rashes, diarrhoea, arthritis, and general failure to thrive [30,138]. Elements of both conditions have been recapitulated in mouse models. CYLD deficiency leads to disturbances in lymphocyte development, proliferation, and responsiveness, leading to mild inflammation and susceptibility to carcinogen-induced tumour formation [139-144]. In contrast, mice expressing two different point mutant alleles of OTULIN, that encode a catalytically compromised protein, display embryonic lethality around E12.5-E14, which is thought to be caused by defective Wnt signaling [120]. Conditional or cell type-specific OTULIN deletion causes severe, systemic inflammatory phenotypes associated with increased signalling and cytokine release from macrophages [30]. A further knock-in mouse model expressing a catalytically inactive form of OTULIN (C129A) [145], dies during embryogenesis (E10.5), due to aberrant cell death. The phenotype resembles both germ line mutation of OTULIN and loss of LUBAC components, confirming LUBAC deubiquitylation as a significant physiological function of OTULIN. Furthermore, it suggests that cell death may contribute to systemic inflammation in mice and humans with OTULIN defects [30,120,138,145-147]. The C129A knock-in mutation converts OTULIN into a high affinity ubiquitin binding domain, which binds and protects Met1-linked chains [117,119,148]. In

ORAS/Otulipenia, decreased stability of mutant OTULIN leads to reduced protein levels. These two settings may respectively block and enable, compensatory CYLD recruitment to LUBAC, which could account for the different severity of phenotypes.

The OTU family DUB, A20 (TNFAIP3), is induced by NF κ B following activation of pattern recognition and cytokine receptors [149,150]. It possesses distinct binding domains for both Lys63- and Met1 chains [151], yet strongly favours cleavage of Lys48 linkages *in vitro.* However, in cells it can become phosphorylated, which further stimulates Lys48 activity and unleashes otherwise latent Lys63 directed activity [20,124,152,153]. In distinction to CYLD, it is equally active towards branched Lys48-Lys63 chains, which have also been linked to the NF κ B pathway [154]. Mouse models expressing a catalytic site mutation of Cys103 to Ala [153,155,156], do not fully replicate the phenotype of A20 loss [157]. Consistent with this observation, is the finding that A20 is unable to cleave Met1-linked ubiquitin chains [20,26], yet regulates Met1 signalling. Recruitment of A20 to immune receptor signaling complexes suppresses NF- κ B signaling in a catalytically independent manner. The primary effect of A20 is to proposed to reflect binding and sequestration of linear ubiquitin chains via its ZnF7 domain [133].

OTUD4 is nominally a Lys48 chain-linkage specific DUB that interacts with the Toll Like receptor interacting protein MyD88. However in cellular extracts OTUD4 shows selectivity for Lys63 linkages. Accordingly, OTUD4 opposes Lys63-linked ubiquitin modification of MyD88 and also limits NF κ B signaling. Notably, selectivity for Lys63 is conferred by OTUD4 phosphorylation [158]. Such DUB "linkage switching" by post-translational modification is an interesting new concept, that might be more widely adopted.

Endosomal DUBs

Activated receptor tyrosine kinases, such as EGF Receptor (EGFR), become ubiquitylated and undergo endocytosis. Upon reaching early/sorting endosomes, ubiquitylation is used to direct receptors towards the lysosomal pathway, using the ESCRT (EndoSomal Complex Required for Transport) machinery [159]. Mass spectrometry analysis has shown that EGFR is ubiquitylated at multiple sites, with Lys63 being both the predominant chain linkage type and required for efficient sorting [160,161]. The ESCRT-0 complex, comprising HRS/HGS and STAM, provides the first point of engagement of ubiquitylated receptors with the ESCRT machinery [159]. The non-selective DUB, USP8, and the stringent Lys63 selective metalloprotease AMSH (STAMBP), compete for binding to STAM, recalling the competition between CYLD and OTULIN for LUBAC binding described above. Each DUB also binds a palette of ESCRT-III components via their respective N-terminal MIT domains [162]. Recent findings suggest that USP8 controls the ubiquitylation state of the ESCRT-III component CHMP1B and may promote its assembly into a membrane associated ESCRT-III polymer [163]. EGF stimulates CHMP1B ubiquitylation and also promotes USP8

recruitment to endosomes [163,164]. Perhaps the deubiquitylation of CHMP1B represents a check-point governing the temporal and spatial assembly of the ESCRT-III polymer which promotes budding of EGFR laden vesicles into the lumen of the endosome/multivesicular body. A further key function of USP8 catalytic activity is to deubiquitylate and stabilise ESCRT-0, which is otherwise degraded by the proteasome [164]. The robustness of this finding is supported by data from CRISPR/Cas9 cell viability screens which show a correlation in sensitivities between loss of either USP8 or the ESCRT-0 component HRS [76].

The Lys63 directed activity of AMSH is unable to compensate for USP8. However, AMSH can influence receptor fate; for example, it promotes the recycling of activated EGFR [165]. It remains an open question whether the stringency of AMSH, or its close relative AMSHLP (STAMBPL), for Lys63-linked chains may reflect undiscovered roles in specific cell signaling pathways. Intriguingly, loss of function mutations of AMSH, either in the MIT or catalytic domain, lead to Microcephaly Capillary Malformation Syndrome (MIC-CAP) [166,167]. Activating mutations in USP8 lead to Cushing's disease that is characterised by pituitary corticotroph adenomas [168,169]. The interplay between endosomal DUBs associated with the endo-lysosomal degradation pathway parallels aspects of proteasomal DUBs discussed above. Endosomal and proteasomal DUB activities can both reprieve proteins, but are also required to recycle ubiquitin following commitment towards the respective degradation pathways [170].

Lys6 chains, phospho-ubiquitin and the role of DUBs in mitophagy

The selective autophagy of organelles or protein aggregates can be mediated by distributed ubiquitin chains which generate the avidity for low affinity adaptor molecules that link the autophagic cargo to the autophagic membrane [171]. This provides an opening for DUBs to regulate autophagy, the third major pathway of protein degradation, in addition to the lysosomal and proteasomal pathways [172]. The selective clearance of damaged mitochondria (mitophagy) has elicited much interest, particularly since the process can be driven by two Parkinsons disease linked genes PINK1(PARK6) and Parkin (PARK2) [173].

The E3 ligase Parkin preferentially, but by no means exclusively, generates Lys6-linked chains upon its activation at damaged mitochondria [173-175]. Inhibitory auto-ubiquitylation of Parkin with predominantly Lys6 chains is proposed to be contained by the non-specific USP8, which enables Parkin recruitment to mitochondria [176]. USP30 localisation is confined to mitochondria and peroxisomes courtesy of a trans-membrane domain and adjacent polybasic residues [33,177]. It too, shows selectivity for Lys6 ubiquitin linkages and is suggested to restrict Parkin ubiquitylation of some proteins (notably TOMM20) and thereby limit mitophagy [28,29,175,178-180]. Parkin activity is controlled by PINK1, that phophorylates ubiquitin at Ser65 the UBL domain of Parkin itself at an equivalent position [181-183]. Parkin is recruited to mitochondria by this phospho-ubiquitin and creates more PINK1 substrate by further ubiquitylating mitochondrial proteins in a feed-forward

loop. This represents the physiological context where the role of ubiquitin phosphorylation is best understood, although other phosphorylation sites on ubiquitin have also been identified [184].

How does phosphorylation of ubiquitin influence DUB activity and selectivity? Chains assembled from Ser65 phosphoubiquitin provided poor substrates for a panel of 12 DUBs, with few exceptions [180]. In a separate study 20 isomeric dimers of phosphoubiquitin, with phosphorylation at Ser20, Ser57 or Ser65 were profiled against 31 DUBs, most of which were less able to cleave the phosphoubiquitin dimers than their unphosphorylated counterparts [185]. This finding is particularly pronounced for Ser65 phosphorylation of ubiquitin and is accounted for by structural considerations [180,185]. In the case of USP30, Ser65 phosphorylation impairs activity against Lys6 and other chain types [28,180]. Structural and biochemical analysis of Lys6-linked ubiquitin dimer processing reveals that phosphorylation of the distal ubiquitin but not the proximal ubiquitin is incompatible with USP30 engagement. In fact, in a tetra-ubiquitin molecule a single phosphorylation of the distal ubiquitin is sufficient to hinder hydrolysis to a similar extent as the fully phosphorylated form. Thus, at mitochondria, PINK1-dependent phospho-capping of Lys6-ubiquitin chains will generate a DUB-resistant mitophagy signal, preserving recruitment sites for Parkin and adaptor proteins that link the mitochondria to autophagosomal membranes. For this reason, recent models have proposed a role for USP30 upstream of PINK1, by limiting initial PINK1-substrate availability and setting the threshold for PINK1 dependent mitophagy [28,33,186].

DUB control of Lys11 chains and the cell cycle

Multiple DUBs have been linked to different stages of the cell cycle, but it is during mitosis that linkage selectivity appears to be most critical [187]. The onset of anaphase is governed by activation of the E3 ubiquitin ligase complex, APC/C, which promotes the degradation of CyclinB and Securin. Thereafter, it targets multiple substrates until it again becomes inactive at the end of G1. In metazoa, APC/C teams up with UBE2C to build short chains linked by Lys11, Lys48 or Lys63 molecules onto substrates and then with UBE2S to extend and branch existing chains with Lys11 linkages [188-191]. UBE2S branching activity has been shown to be required for efficient proteasomal degradation of various substrates [53,192]. The OTU family member Cezanne is the most prominent Lys11 specific DUB and accumulates during mitosis [20,24,193,194]. It has been shown to control the degradation kinetics of some (e.g Cyclin B and Securin) but not all APC/C substrates during mitotic progression [194]. Moreover, depletion of Cezanne leads to accumulation of micronuclei during mitosis which can be reversed by co-depletion of UBE2S. Interestingly, Cezanne is amplified in >30% of breast tumours and is situated within an amplicon that lacks a verified oncogene [195].

DUBs as analytical tools

A suite of DUBs with defined chain linkage specificities provides a useful analytical tool to analyse ubiquitin chain architectures by parallel electrophoretic analysis of enzyme treated samples. In the first instance, one can use a promiscuous DUB, e.g. USP2, to show that a protein is indeed ubiquitylated [196]. Further analysis of banding patterns, following treatment with selective DUBs, allows the estimation of linkage types associated with a particular protein substrate. By analogy with restriction digests used in molecular biology, this has been termed ubiquitin chain restriction (UbiCRest) analysis [197]. UbiCRest analysis enables first insights into the architecture of heterotypic ubiquitin chains. One elegant example combined USP2 (non-specific), OTULIN (Met1-Ub specific) and AMSHLP (Lys63 specific) to dissect the ubiquitin chain linkages associated with innate immune signaling components and led to the discovery of the presence of heterotypic chains consisting of Met1 chains built upon a Lys63 linked scaffold [116].

DUBs as therapeutic targets

Linkage of DUBs to the stability of specific client proteins has offered a means to extend the druggable proteome (Table S2)[198]. In a nutshell, for any protein turned over in a ubiquitin dependent fashion, inhibition of it's cognate DUB may lead to protein destabilisation. High value oncology targets linked to DUBs include MYC (USP28, USP36, USP37), NMYC (USP7), MDM2/p53 (USP7) and MCL-1 (USP13,USP9X) [199-203]. Many small molecule DUB inhibitors have been reported in the literature, but until recently few of these have been specific [26].

The response to DNA damage is controlled by the tumour suppressor gene and transcription factor p53, which can promote either apoptosis or cellular senescence. The short half-life of p53, typically around 10 minutes, enables rapid adjustment in protein levels through changes to turnover kinetics. The most prominent E3-ligase associated with p53 is MDM2, whose own stability is governed by autoubiquitylation. Under basal conditions, USP7 binds to MDM2 and rescues it from degradation, indirectly reducing p53 levels [204]. In the last year, several publications have reported highly specific USP7 inhibitors that all elevate p53 levels [205-208]. Although, USP7 inhibitors retard tumour growth in a mouse xenograft model, available evidence suggests that this is independent of p53 status [207,209]. MDM2 is just one of many physiological substrates linked to USP7, which include other proteins linked to tumour growth such as PTEN, FOXP3 and Claspin [210-212]. One inhibitor developed by Genentech from fragment screening binds 12Å from the catalytic centre and impedes binding of the distal ubiquitin of the favoured Lys48-linked substrates [205]. The three other studies converge on small molecules with a shared core structure, making identical key contact sites, as revealed by high resolution crystal structures [206-208]. Interestingly, these critical contact residues are conserved in other USP family members. The exquisite specificity of these compounds arises from a unique USP7 configuration in its ubiquitin-unbound form [213]. In this apo-form, the catalytic triad essential for hydrolysis is misaligned and a cleft between structural domains is rendered compatible for compound binding about 5Å from the

catalytic cysteine. These studies have generated tool compounds for acute manipulation of USP7 activity that may inform clinical positioning of candidate drug molecules.

Inhibition of the 19S proteasome regulatory particle DUB, PSMD14 suppresses proteasome activity. The development of specific PSMD14 inhibitors is at an early stage, but a proof of principle has been established [214]. Successor compounds may offer therapeutic alternatives to the established inhibitors of active sites in the 20S core particle, such as Bortezimib, which are used to treat multiple myeloma. In contrast inhibition of another 19S regulatory particle DUB, USP14, enhances the degradation rate of certain proteins linked to neurodegeneration, such as the Alzheimer's Disease linked Tau and Prion proteins [215-217]. The USP14 inhibitor (IU1) occupies a similar cleft in the structure to several of the USP7 inhibitors, albeit with a different orientation [206-208,218]. All these compounds block access of the ubiquitin C-terminus to the catalytic centre. Inspection of the patent literature suggests that similar breakthroughs have been made for further DUBs of therapeutic interest [198]. The emerging picture suggests that the conformational plasticity of the USP catalytic domain frequently offers opportunities for selective inhibition.

Conclusions

At least a third of active DUBs have now been assigned some level of specificity with regard to their action on ubiquitin chains or ubiquitin like modifiers. Alongside this, information on copy numbers and localisation, has begun to provide a composite outline of their collective impact on the cellular distribution of ubiquitin. We now appreciate that complex cellular processes such as DNA repair and innate immune signaling rely on co-ordination between different ubiquitin chain linkage types, that is facilitated by DUBs with cognate specificities (e.g. Met1, Lys48, Lys63). However, our understanding of the biology associated with some chain linkage types remains very limited and further levels of complexity (post-translational modification of ubiquitin, branched chains) are presenting new frontiers. Knowledge of specificity and discovery of new DUB activities have led to their adoption as analytical tools. Association of individual DUBs to key pathways in oncology, immunity and neurodegeneration are driving drug discovery programmes that have rendered the first generation of highly specific inhibitors.

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cleavage type	DUB	Refs
Lys63	AMSH, AMHLP, BRCC36, OTUD1, ZUP1, CYLD, pOTUD4	65,124,219,220, 20, 15-18, 13, 27,124, 158
Lys48	OTUB1, OTUD4, A20, MINDY	20,13
Lys29/33	TRABID	221
Lys11	Cezanne	24,193
Lys6	USP30	28, 29,175
Linear	OTULIN, CYLD	119,120
non-specific	most USPs (e.g. USP2, USP21)	25
en-bloc	PSMD14, USP14, UCHL5?	64,68,69
free chains	USP5, USP3, USP16	47,222, 223
histones (monoUb)	MYSM1, USP3, USP16, USP22, BAP1	81,80,82,78
NEDD8	COPS5	23
SUMO	USPL1	21
ISG15	USP18	22

Table 1: Summary of known ubiquitin chain linkage or other modification preferences of DUBs reported in the literature. The table indicates DUBs for which biochemical evidence indicates a significant degree of selectivity between chain architectures for the isolated enzyme or embedded within multi-protein complexes. Note that this does not imply absolute stringency in all cases.

Figure Legends

Figure 1 - Major Roles of DUBs

DUBs have key roles in maintaining protein homeostasis and signaling in cells by; (**a)** removing non-degradative ubiquitin (Ub) signals which may regulate protein function directly or contribute to the assembly of multi-protein signalling complexes; (b) rescuing proteins from either proteasomal or lysosomal degradation, (c) maintaining ubiquitin levels by recycling ubiquitin from proteins that are committed to degradation (d) and (e) post-processing following *en bloc* ubiquitin chain removal to maintain free ubiquitin levels, (f) generation of newly synthesised ubiquitin by releasing monomeric ubiquitin from multimeric precursor proteins encoded by four genes. *UBB* and *UBC* encode multiple copies of ubiquitin that are transcribed and translated as linear fusion proteins with a C-terminal extension of one or two amino-acids (shown in pink). *UBA52* and *UBA80* yield ubiquitin fused to the amino terminus of two ribosomal subunits, 40S ribosomal protein L40 (L) and 60S ribosomal protein S27a (S). Thus DUBs are also indirectly involved in ribosome biogenesis.

Figure 2 - Phylogenetic conservation of DUBs across model organisms

DUBs are arranged according to a bootstrapped neighbour joining phylogenetic analysis of their catalytic domains with the most reliable nodes (supported by bootstrap values of >50%)
indicated by a black dot (see 10 for further detail). The following newer members were curated and added manually: OTULIN, FAM105A, the MINDY family, ZUP1, ALG13. A single representative member of the expanded USP17 family is shown (USP17L2). DUBs annotated with * are predicted to be inactive based on sequence or structural considerations. Note that zebrafish MINDY4B is predicted to be active (personal communication, Kay Hofmann). Blue bars indicate human sequences, purple, green and yellow bars indicate the presence of a clearly identifiable orthologue in zebrafish (D. rerio), fly (D. melanogaster) or in either one of two commonly used yeast species (S.pombe and S.cerevisiae) respectively. In the latter case some orthologues cannot be directly assigned to one or the other paralogue (e.g. MINDY1/2). DUBs that have a discernible orthologue in yeast, are indicated in red. Note these include all the essential DUBs shown in Figure 4.

Figure 3 -Sub-cellular localisation of DUBs in mammalian cells.

DUBs which are predominantly localised to the nucleus or with clearly identifiable sub-cellular structures are shown. Data are derived from a systematic sub-cellular localisation screen in HeLa cells 31 combined with individual studies collated here to supplement this overview. pm, plasma membrane; mvb, multivesicular body; ee, early endosome; er, endoplasmic reticulum; go, Golgi;

mt, microtubules; mito, mitochondria; sg, stress granules; ld, lipid droplets; ce, centrosome; nu, nucleus; n, nucleolus; po, peroxisomes.

Figure 4 - Essential DUBs

Seven DUB family members show a consistently high dependency score across multiple genome wide CRISPR and RNAi screens, comprising data from more than 400 cell lines, meaning that they are required for cell viability in nearly all cell types (Table S1). (a) USP39 and JAMM family member PRPF8 are both catalytically inactive (Pseudo-DUBs) and cooperate in pre-mRNA splicing. USP39 is a component of U4/U6-U5 tri-snRNP, which is a key building block of the spliceosome and requires PRPF8 for its assembly. (b) The two JAMM family members PSMD14 (active) and PSMD7 (inactive) form *a* functional unit within the lid of the 19S proteasome regulatory particle that removes ubiquitin from proteins committed to degradation. (c) COPS5 and COPS6 make up another DUB-pseudoDUB pair belonging to the JAMM family, that forms the enzymatic core of the eight subunit multiprotein complex COP9-signalosome (CSN), which catalyses the removal of the Ubiquitin-like protein NEDD8 from the Cullin component of Cullin-Ring E3 ligases (CRL). Modification of the Cullin scaffold subunit with NEDD8 (N; known as neddylation) on a conserved lysine is required for CRL activation. It results in a reorientation of RING Box 1 (Rbx1) that facilitates ubiquitin transfer from a RING-bound E2 (not shown) onto a substrate recruited to the substrate receptor (R) that is linked via an adaptor (A) to the Cullin. R, substrate receptor; A, adaptor; Rbx, Ring box protein that recruits the E2. (d) The ZnF UBP domain of USP5 (also called Isopeptidase T) specifically recognises a glycine at the unconjugated carboxy-terminal of ubiquitin and specialises in the generation of free ubiquitin by disassembly of unanchored ubiquitin chains.

Figure 5 - DUBs implicated in the double strand break (DSB) DNA damage response (DDR)

(a) USP22 is a DUB component of the SAGA complex, a multi-enzyme transcription co-activator complex, that functions in DDR to limit H2B(Lys120) by RNF20/40 E3-ligase. This ubiquitin modification is proposed to promote chromatin relaxation, required for the recruitment of the repair machinery.

(b) DSBs activates ATM kinase (not shown) which phosphorylates both H2AX and the DDR scaffold protein Mediator of DNA damage checkpoint protein 1 (MDC) leading to recruitment of the E3, RNF8 which together with the E2 UBC13 generates Lys63-linked ubiquitin chains on either L3MBTL2 or Histone H1. These Lys63-linked chains (depicted in purple) can be removed by BRCC36 (also known as BRCC3), which forms a functional unit with the catalytically inactive MPN-like ABRAXAS1 protein within the BRCA1-A complex that is recruited via the RAP80 subunit. Their formation can also be suppressed by OTUB1 inhibition of UBC13 that is independent of catalytic activity. Lys63-linked chains recruit a second E3 ligase, RNF168, which

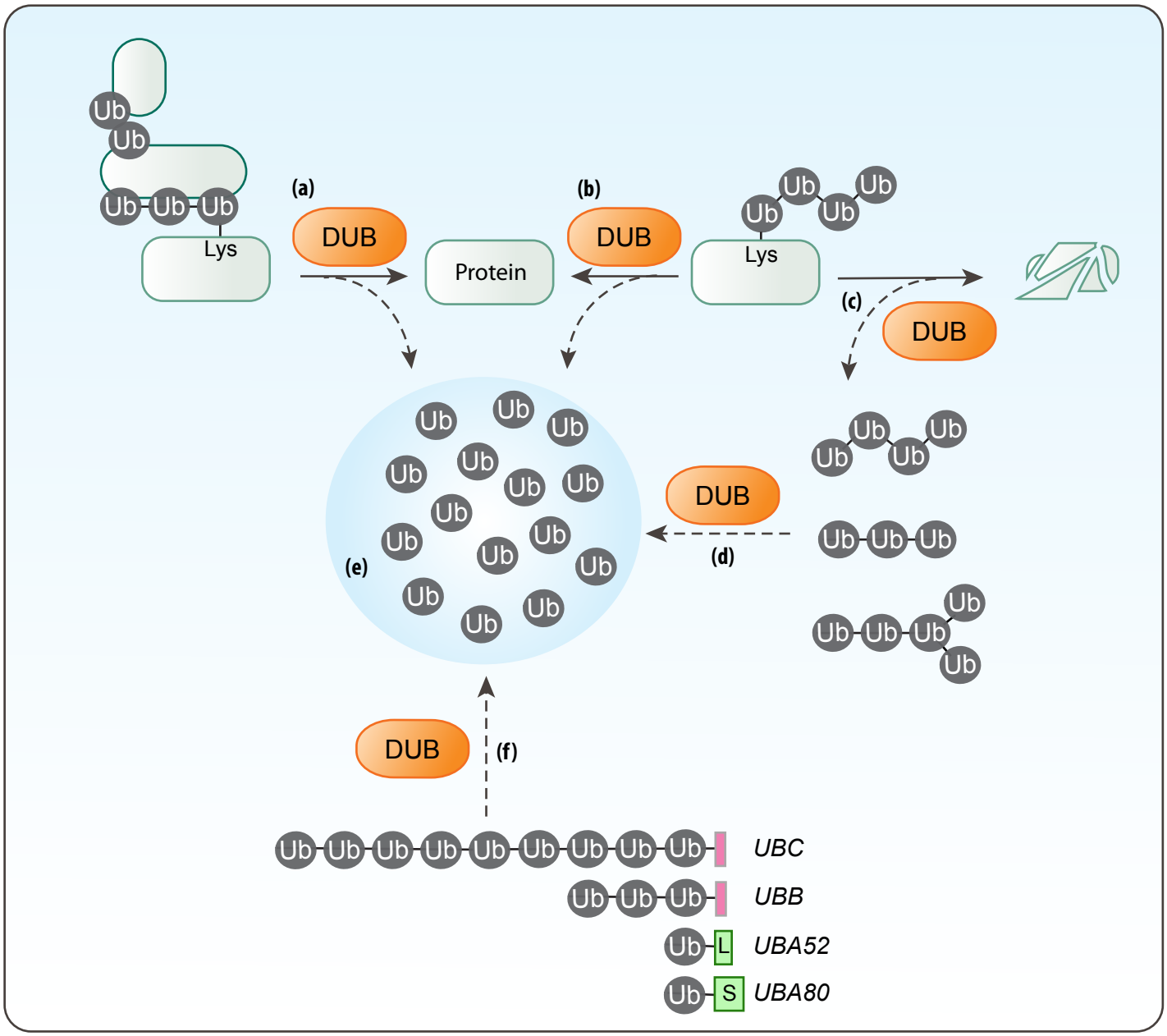
in conjunction with UBCH5 mono-ubiquitylates H2A on Lys13 and Lys15. This modification that is opposed by USP51, recruits p53BP1 and is required for DNA repair via non-homologous end joining (NHEJ).

(c) Cezanne disassembles Lys11-linked ubiquitin chains that are generated by RNF8 in conjunction with the E2 UBE2S on damaged chromatin (including H2A) and regulate transcriptional silencing.

(d) USP48 opposes the BRCA1/BARD1 ubiquitin E3 ligase that ubiquitylates H2A at Lys 125/127/129 and promotes DNA end resectioning that is necessary for homology directed repair (HDR).

Figure 6 - Chain specific DUBs orchestrate innate immune signalling

Activation of innate immune signalling receptors (ie pattern recognition (e.g.TLR4, NOD2) or cytokine receptors (e.g. TNFR and IL-1R)) involves the assembly/disassembly of both Met1 (linear), and Lys63-linked chains on components of the primary receptor signaling complexes. The linear ubiquitin specific LUBAC E3 ligase complex (HOIP, HOIL-1 and Sharpin), is responsible for the assembly of Met1-linked chains (blue) on adapters, or on existing Lys63-linked chains (purple) generating branched or hybrid Lys63-Met1chains. It also undergoes auto-ubiquitylation. Met1 chains mediate downstream signaling by interaction with the inhibitor of nuclear factor kappa-B kinase (IKK) sub-unit NEMO and subsequent activation of IKK via phosphorylation by TAK1, which is recruited via Lys63-linked chains Two DUBs engage LUBAC via the same PUB domain of HOIP: OTULIN, a stringent Met1-specific OTU DUB binds to LUBAC directly, whereas the USP, CYLD binds via an adapter SPATA2 and is able to disassemble both Lys63 and Met1-linked chains. OTUD4 is an intrinsically Lys48-specific DUB which is converted into a Lys63-specific DUB to remove such chains from MyD88, an adapter (component) of the signalling complex (not shown). Likewise, A20, another member of the OTU family of DUBs acquires Lys63-linked ubiquitin chain processing activity upon phosphorylation. In addition, A20 encodes a series of zinc fingers (ZnFs) that bind and sequester Met1- or Lys63-linked ubiqutin chains.


Ub
Ub
Ub
Ub
Ub
Lys
(a)
DUB
Protein
(b)
DUB
Ub
Ub
Ub
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Lys
(c)
DUB
Ub
Ub
Ub
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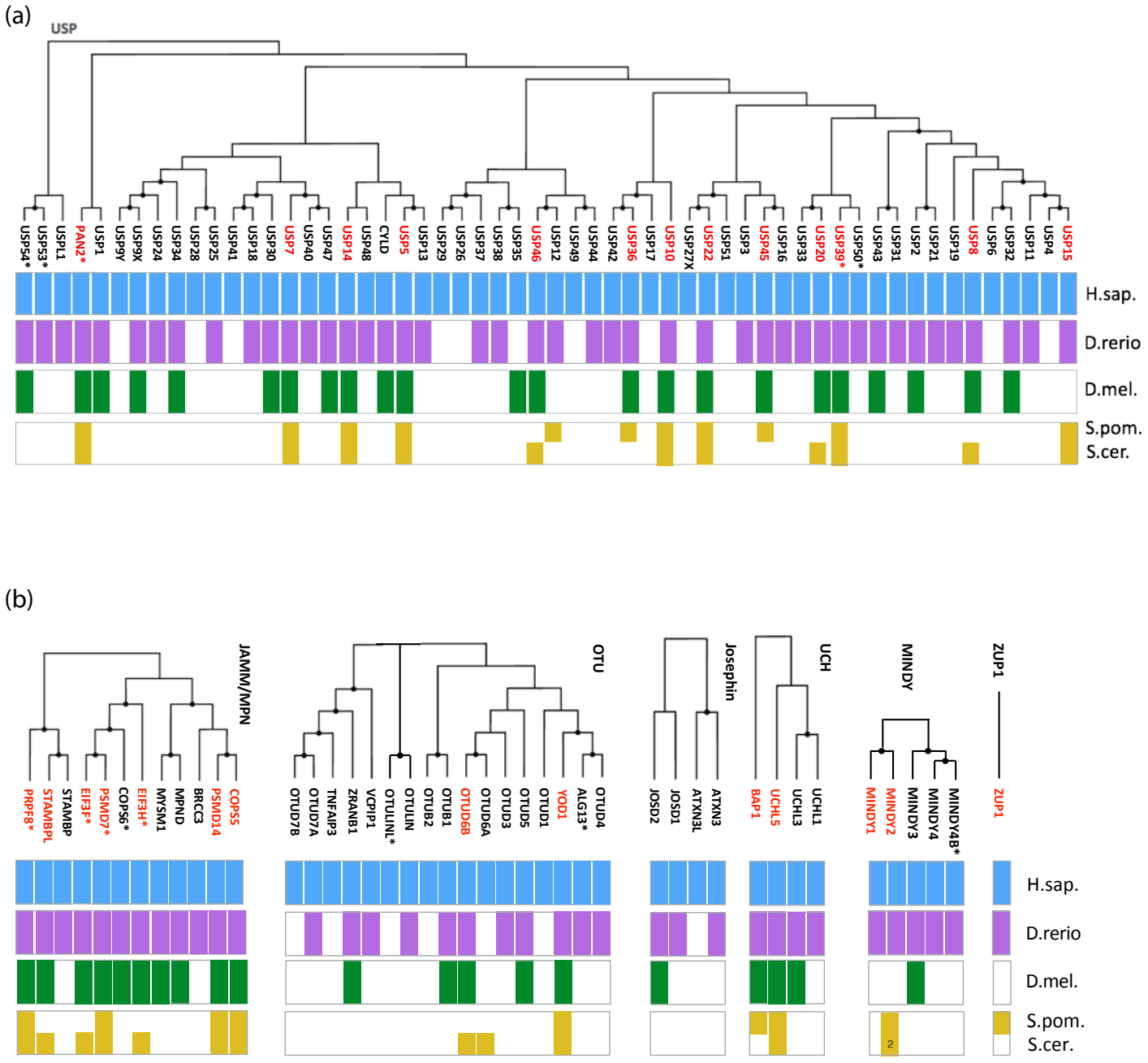
UBC
Ub
Ub
Ub
UBB
Ub
L
UBA52
Ub
S
UBA80

Figure 1


(a)
USP
USP54*
USP53*
USPL1
PAN2*
USP1
USP9Y
USP9X
USP24
USP34
USP28
USP25
USP41
USP18
USP30
USP7
USP40
USP47
USP14
USP48
CYLD
USP5
USP13
USP29
USP26
USP37
USP38
USP35
USP46
USP12
USP49
USP44
USP42
USP36
USP17
USP10
USP27X
USP22
USP51
USP3
USP45
USP16
USP33
USP20
USP39*
USP50*
USP43
USP31
USP2
USP21
USP19
USP8
USP6
USP32
USP11
USP4
USP15
H.sap.
D.rerio
D.mel.
S.pom.
S.cer.
(b)
JAMM/MPN
PRPF8*
STAMBPL
STAMBP
EIF3F*
PSMD7*
COPS6*
EIF3H*
MYSM1
MPND
BRCC3
PSMD14
COPS5
OTU
OTUD7B
OTUD7A
TNFAIP3
ZRANB1
VCPIP1
OTULINL*
OTULIN
OTUB2
OTUB1
OTUD6B
OTUD6A
OTUD3
OTUD5
OTUD1
YOD1
ALG13*
OTUD4
Josephin
JOSD2
JOSD1
ATXN3L
ATXN3
UCH
BAP1
UCHL5
UCHL3
UCHL1
MINDY
MINDY1
MINDY2
MINDY3
MINDY4
MINDY4B*
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ZUP1
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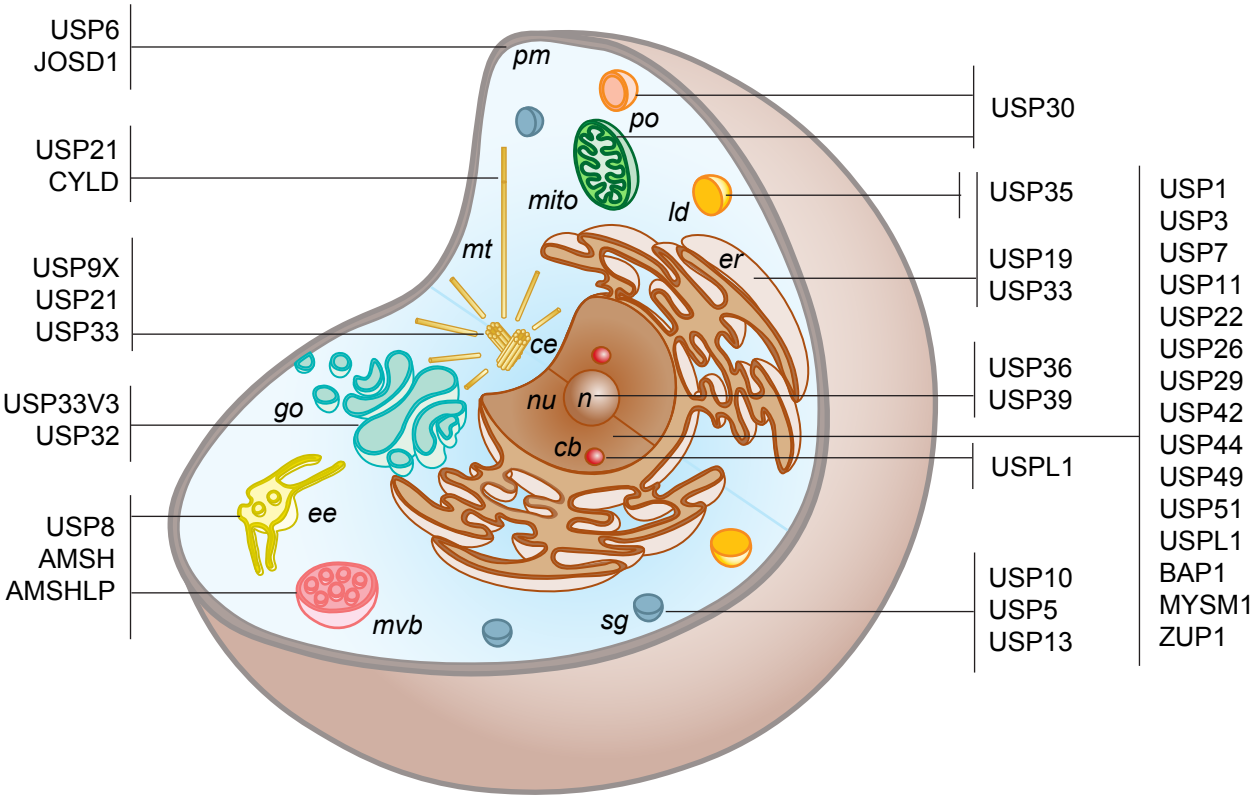
D.rerio
D.mel.
S.pom.
S.cer.

Figure 2


USP6
JOSD1
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CYLD
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USP21
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pm
po
mito
ld
mt
er
ce
nu
n
cb
go
ee
mvb
sg
USP30
USP35
USP19
USP33
USP36
USP39
USPL1
USP10
USP5
USP13
USP1
USP3
USP7
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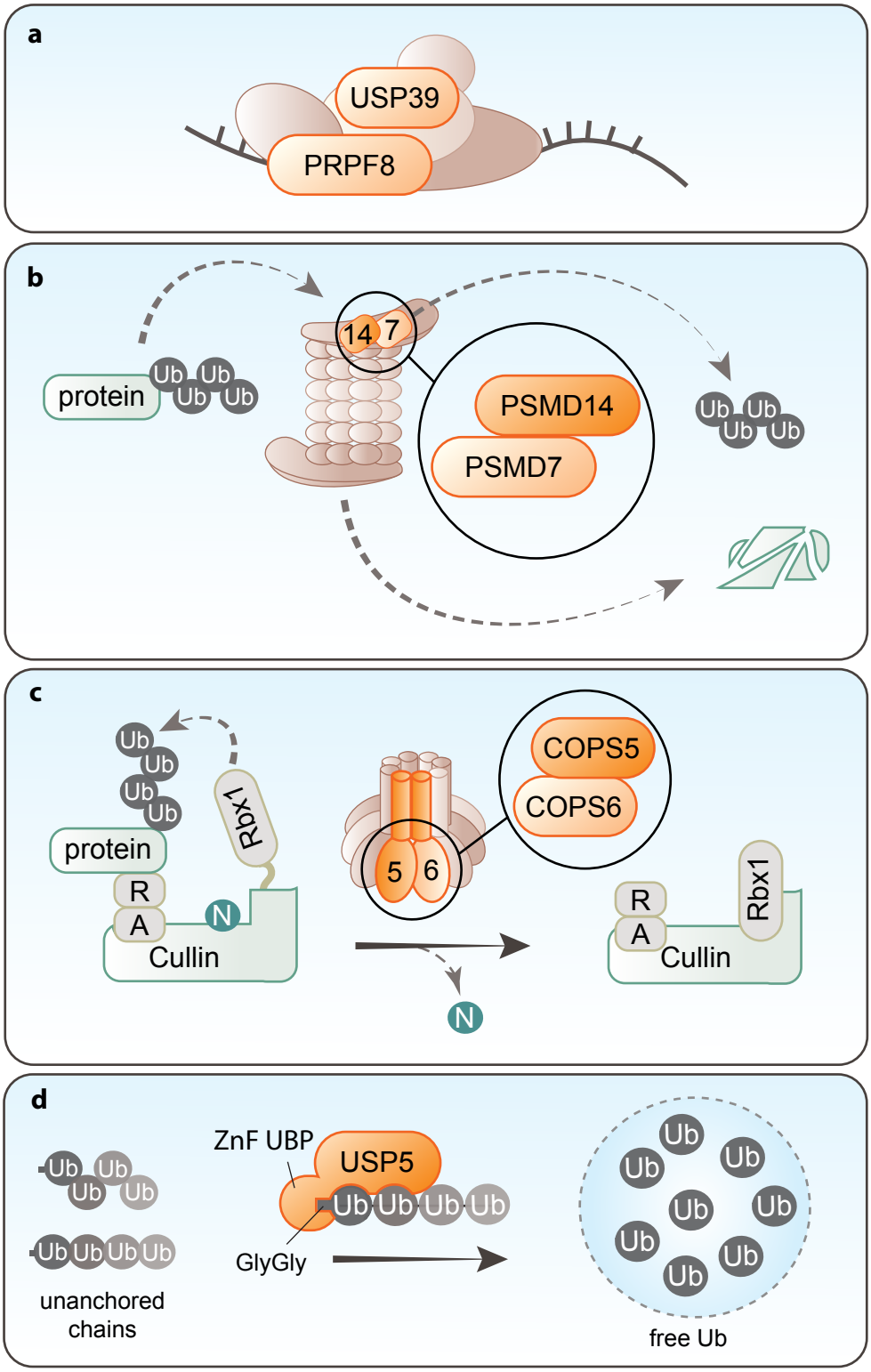
USPL1
BAP1
MYSM1
ZUP1

Figure 3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USP39
PRPF8
b
protein
Ub
14
7
PSMD14
PSMD7
c
protein
Rbx1
R
A
N
Cullin
COPS5
COPS6
5
6
d
unanchored chains
ZnF UBP
USP5
GlyGly
free Ub

Figure 4

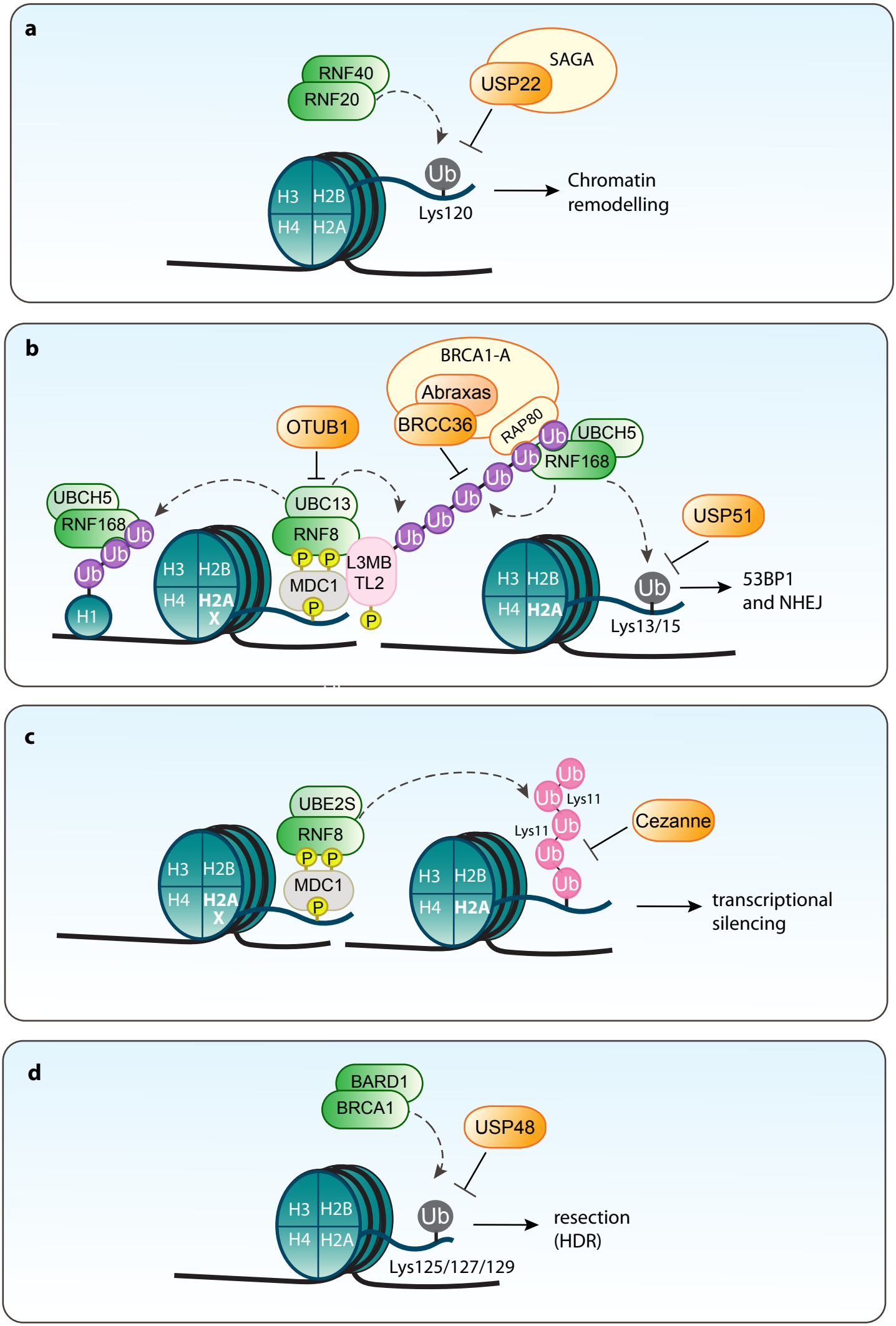
a
RNF40
RNF20
SAGA
USP22
Ub
Lys120
H3
H2B
H4
H2A
Chromatin
remodelling
b
BRCA1-A
Abraxas
BRCC36
RAP80
OTUB1
UBCH5
RNF168
Ub
UBC13
RNF8
P
MDC1
L3MB
TL2
H1
H2A
X
USP51
Lys13/15
53BP1
and NHEJ
c
UBE2S
RNF8
MDC1
Lys11
Cezanne
transcriptional
silencing
d
BARD1
BRCA1
USP48
Lys125/127/129
resection
(HDR)

Figure 5

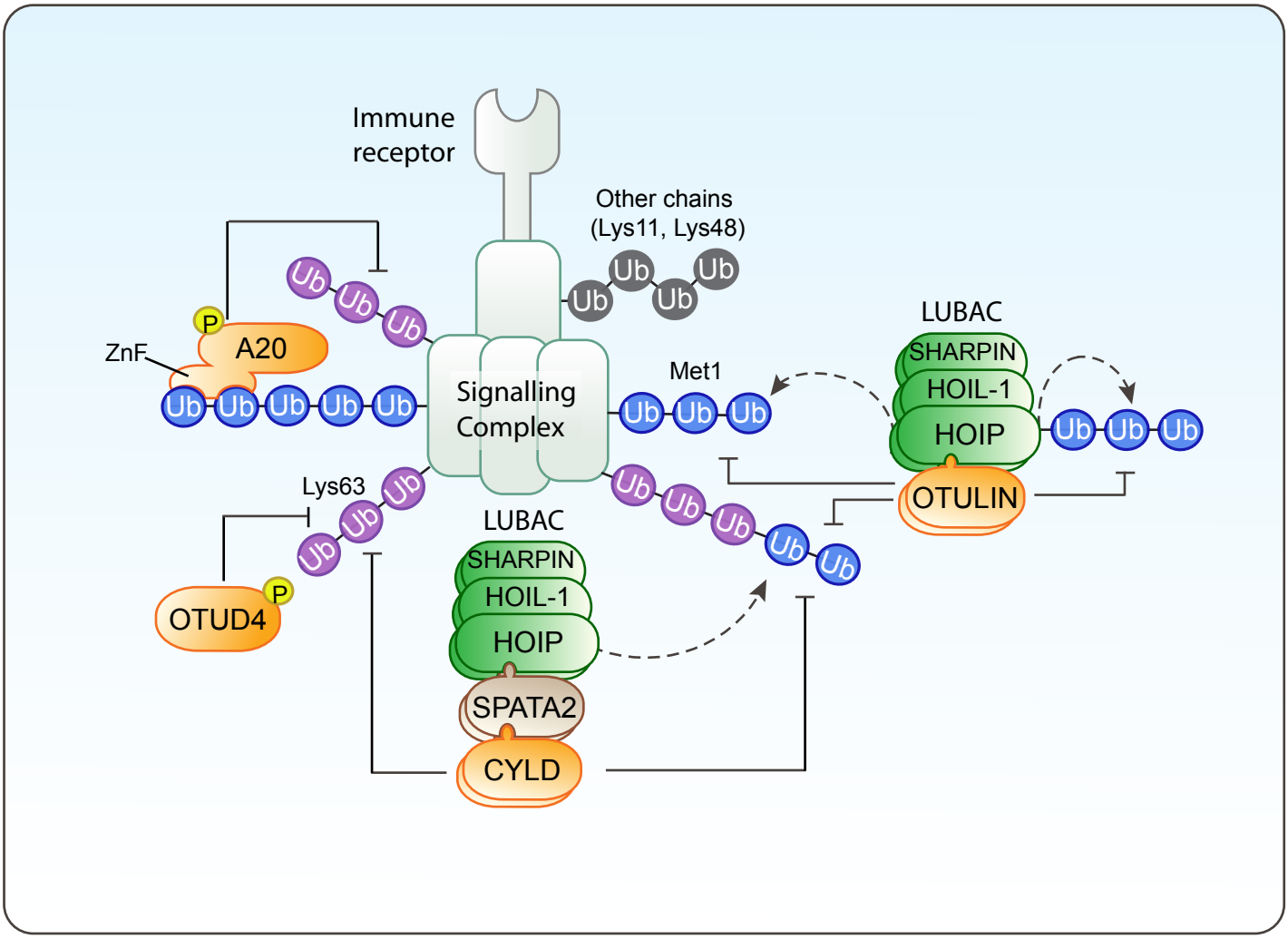
Immune
receptor
Other chains
(Lys11, Lys48)
Ub
Ub
Ub
Ub
Ub
P
A20
ZnF
Ub
Ub
Ub
Ub
Ub
Signalling
Complex
Met1
Ub
Ub
Ub
LUBAC
SHARPIN
HOIL-1
HOIP
Ub
Ub
Ub
OTULIN
Lys63
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
LUBAC
SHARPIN
HOIL-1
HOIP
SPATA2
CYLD
OTUD4
P

Figure 6

	Hart et al. (17) *BF ≥ 6*	Broad Avana (436) *CERES <-1*	Broad Avana (436) *CERES <-0.5*	Broad GeCKO (33) *CERES <-1*	Broad GeCKO (33) *CERES <-0.5*	Wang et al. (14) *CERES <-1*	Wang et al. (14) *CS <-0.5*	McFarland RNAi (713) *DEMETER 2 <-0.5*
PSMD14	94	99	100	100	100	100	100	81
COPS6	94	91	100	55	100	36	100	95
PRPF8	88	99	100	100	100	100	100	98
USP39	88	0	44	15	100	86	100	100
USP5	82	61	99	3	94	100	100	54
COPS5	82	76	100	0	15	7	93	91
USPL1	82	15	96	91	100	0	50	59
EIF3H	71	13	93	3	85	0	36	68
PSMD7	65	100	100	100	100	14	100	99
USP36	65	58	100	0	58	0	79	1
BAP1	59	1	56	3	82	0	93	5
USP7	53	9	61	36	94	0	79	24
USP37	53	0	53	0	61	0	86	1
USP8	47	6	87	0	15	0	79	21
EIF3F	47	7	94	0	76	0	36	95
High >80%								
Low 50-80%								
No <50%								

Supplemental Table 1: Core fitness DUBs

DUBs that show the most consistent core-dependency across several high quality genome wide CRISPR screen analysis datasets [1-5] and a combined analysis of multiple comprehensive RNAi screens (DEMETR2) [6]. Numbers of cell lines included in each analysis are indicated in brackets.Table indicates the percentage of cells that show dependency based on a threshold score shown in italics in the column header. A high degree of dependency (>80% cell lines) is shown in red. For the Hart et al. dataset, we chose the recommended strict Bayes Factor threshold score of ≥ 6. A CERES score of -1 for Broad Avana (2018Q2) [2,4] and Broad GeCKO [1] datasets is comparable to the median of all pan-essential genes. The DEMETER2 dependency score used by McFarland et al., reflects shRNA depletion values taking off-target seed effects into account, where a score of -1 is indicative of essentiality, based on negative and positive control gene sets used for scaling [6]. The CS score used in the acute myeloid leukemia-cell focused screen (Wang et al., [5]), is defined as "the average log2-fold change in abundance of all sgRNAs targeting a given gene between initial and final cell populations" [5]. Genes shown in bold are contained within the Core essential gene dataset (CEG2) as defined in [3]. See also Figure 4.

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DUBs mutated in disease				
	Disease setting	**Disease association**	**Genetic alterations**	**References**
BAP1	Cancer	Renal cell carcinoma, Uveal Melanoma, Mesothelioma	Loss of function	1-3
CYLD	Cancer, Innate immune signalling	Cylindromatosis	Loss of function	4
OTULIN	Innate Immune signalling Inflammation	ORAS	Loss of function	5, 6
USP8	Adenomas, Cancer	Cushing's Disease	Gain of function	7
USP48	Adenomas, Cancer	Cushing's Disease	Gain of function	8
STAMBP (AMSH)	Developmental disease	Microcephaly-capillary malformation	Loss of function	9

	Indirect extension of druggable proteome through DUB targeting			
	Disease setting	**Disease application**	**Indirect targets**	
USP1	Cancer	inhibitor sensitises cells to cisplatin	FANCD2, PCNA	10
USP7	Cancer	neuroblastoma, immunotherapy	Regulates MDM2/p53, NMYC, FOXP3 levels	11, 12
USP9X	Cancer	-	Reported to stabilise the anti-apoptotic protein MCL1, regulates centrosome duplication	13, 14
USP13			Reported to stabilise the anti-apoptotic protein MCL1 and determine sensitivity to BH3 mimetics	15
USP28	Cancer	MYC driven tumours	FBW7 clients (MYC, Jun etc)	16-18
PSMD14	Cancer	Alternative to established proteasome inhibitors	Positive regulator of proteasome activity	19
USP14	Neurodegeneration	Alzheimers Disease	Limits proteasomal degradation	20-23
USP30	Neurodegeneration	Parkinson's disease	Suppresses Mitophagy	24-27
USP19	Muscle atrophy		Promotes atrophy through enhancement of glucocortoid signaling	28

Supplemental Table 2: Selected DUBs mutated in human disease and/or presenting attractive therapeutic targets.

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